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## ABSTRACT

A preliminary study was made of the effects of chlorpromazine on the incorporation of $P^{32}$ into a number of P-containing fractions of adrenals, lung, liver, kidney, heart and brain of the rat. The fractions studied were the plasma inorganic P, tissue inorganic P, acid soluble P, lipid P and RNA P.

The administration of chlorpromazine caused significant increases in the incorporation of $P^{32}$ into the plasma inorganic P fraction. It is suggested that the increased activity of this fraction was due to a decreased transfer of $P^{32}$ from the plasma to the intracellular fluid. This might, in turn, be due to changes in the permeability of the cellular membrane brought about by chlorpromazine.

Decreases in the incorporation of $P^{32}$ into the lipid P and RNA P fractions of all tissues, were noted after the administration of 50 mg./Kg. of chlorpromazine. However, when the dose of the drug was reduced to 25 mg./Kg. these decreases were no longer apparent. It is suggested that the uncoupling effects of chlorpromazine in oxidative metabolism, which have been demonstrated *in vitro*, are not evident in the intact animal except with doses of the drug larger than normally used therapeutically. Therefore, it is possible that the pharmacological actions of the drug are not due to its uncoupling effects on phosphorylative metabolism.

In view of the relative importance generally ascribed to the effects of chlorpromazine on the metabolism of the adenosine nucleotides, an investigation of the current methods for the separation of these compounds was undertaken. The most suitable method

for the desired purposes was found to be a paper chromatographic method involving the development of the chromatograms using 2 solvents. It was then necessary to develop in two separate directions.

The use of the method for phosphorus estimation described by Lucena-Conde and Prat (105) is also discussed. The combined methods of chromatographic separation and phosphorus estimation have a number of advantages over other current methods.

THE UNIVERSITY OF ALBERTA

# THE EFFECTS OF CHLORPROMAZINE ON PHOSPHORUS METABOLISM IN THE MALE RAT

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

FACULTY OF PHARMACY

by

JACK DIAMOND

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# INTRODUCTION

A remarkable surge of interest in drugs which affect the mental aspects of behaviour resulted from the discovery, in 1947, of the unusual psychotomimetic properties of lysergic acid diethylamide (1). The similar effects of mescaline and certain other agents have stimulated interest in the possibility that abnormal mental states may be directly associated with biochemical or physiological disturbances in the patient.

More recently, the introduction of a group of drugs, useful in the treatment of abnormal psychotic states, has provided further impetus to the study of psychopharmacology. These psychotherapeutic drugs, referred to as tranquilizers or ataraxics, have the ability to produce calmness, tranquility, and amelioration of tension, without excessive drowsiness or deleterious effects on mental performance (2). They include a variety of drugs with widely different chemical structures and, although they have essentially the same effects, it is difficult to assign a common mechanism of action to these drugs. The most prominent members of this group are the Rauwolfia alkaloids, chlorpromazine, meprobamate, and azacyclonol, although other drugs with similar actions are constantly being introduced. A great deal of attention has been given, in recent years, to reserpine and chlorpromazine, both of which have firmly established ataractic properties.

Attempts to elucidate the biochemical effects of chlorpromazine (CPZ) and to relate these to the pharmacological action of the drug have resulted in many studies on oxidative phosphorylation. Important in the considerations have been the

effects of CPZ on the energy supplying molecule adenosine triphosphate.

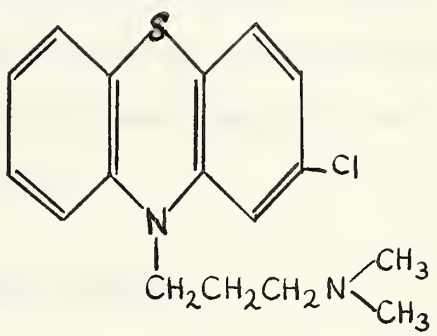
The purpose of this work was to evaluate the effects of CPZ on the phosphorus metabolism of the rat. Of principle interest was the application of a more specific method for the estimation of the phosphorus fractions and the measurement of the rate of incorporation of radioactive phosphorus into these fractions.

# LITERATURE SURVEY

## A. CLINICAL ASPECTS OF CHLORPROMAZINE

Hamon, Paraire and Velluz (3) and Delay, Deniker and Harl (4) were the first to report on the calming effects of CPZ in a group of patients with excitation and agitation consisting of anxious and melancholic states, schizophrenia, delirium, hallucinations and obsessions. Their observations were confirmed and extended by several investigators (5, 6, 7) who in spite of differences in experimental conditions, agreed that CPZ was effective in reducing psychomotor excitation of schizophrenic, manic - depressive and toxic origin.

Chlorpromazine is the 10 (3- dimethylamimopropyl) -2- chloro derivative of phenothiazine. It has the structural formula:




This agent appears to be capable of modifying a large number of metabolic processes (8), but the clinical use of the compound stems from:

1. its effectiveness as an anti-emetic agent
2. its ability to potentiate narcotics, sedatives and anesthetics, and, most important
3. its capacity to alleviate anxiety, tension, and agitation without dulling mental acuity.

The vast number of biochemical processes affected by CPZ and the reactivity of the molecule renders the elucidation of the primary

pharmacological action of the drug extremely difficult.

A variety of undesirable side-effects have been reported with continued use of CPZ. Out of a total of 425 patients studied in one series (9) 45% showed mild to moderate drowsiness and 30%, especially those on high doses, showed hypotension. Jaundice occuring during treatment with CPZ was described by Cohen and Archer (10) and by Stacey et al (11). There is now general agreement that it is of an obstructive type due to a choliangolitic hepatitis as manifestation of a drug sensitivity (12). The overall incidence of jaundice in patients receiving CPZ has been estimated to be 1.5% (12). Allergic skin eruptions and rare cases of fatal agranulocytosis have also been reported (13). The development of Parkinsonism is frequent among patients on large doses of CPZ, and its incidence is estimated to be about 15% (14). Treatment with anti-Parkinsonism drugs such as benztropine methane sulfate (Cogentin, Merck, Sharpe, and Dohme) has been found to control this complication effectively.

## B. EFFECTS ON BIOLOGICAL SYSTEMS

### 1. Distribution of CPZ in Body

Chlorpromazine, labelled with radioactive sulfur, has been shown to accumulate in the brain, particularly the hypothalamus, and also in the lung (15, 16, 17). It seems to be associated with the lipid fraction of the tissue to a much greater degree than with the protein fraction (16).

### 2. Effects on Oxidative Metabolism and Enzyme Systems

In 1955 Abood (18) reported that CPZ uncoupled the oxidative

phosphorylation of brain mitochondria in concentrations not affecting overall oxidation. Desci and Mehes (19) showed marked inhibition of oxidative phosphorylation in brain homogenates, also with concentrations of CPZ which had little or no effect on oxygen consumption. Similar inhibitions of oxidative phosphorylation have been reported by various investigators (20-27).

Berger and co-workers (23) found that CPZ seemed to uncouple oxidative phosphorylation in rat liver mitochondria, whereas it failed to do so under the same conditions with rat brain cortex mitochondria. In a later report Berger concluded that the effects on the drug on brain mitochondria were relatively nonspecific (22). However, Dawkins et al., using a somewhat different system, found that brain mitochondria were quite sensitive to the uncoupling actions of CPZ (24).

Century and Horwitt (20) found that CPZ was capable of depressing both oxygen uptake and inorganic phosphate uptake of rat brain homogenates, but in higher concentrations than normally used therapeutically. Similar depression of oxidative metabolism of brain homogenates has been reported by Bernsohn et al (27), Decsi and Mehes (19) and Messer (28). The drug has also been shown to depress respiration of brain slices (29, 30) and mitochondria (18, 31). Messer (28) concluded that reduced phosphorylation due to CPZ was secondary to the depression of oxidative metabolism.

The increased oxygen consumption of cortical slices due to electrical stimulation or potassium stimulation is especially sensitive to the presence of the drug (32, 33, 19).

CPZ has been reported to inhibit several of the steps of the respiratory chain (22) including DPNH-cytochrome c Reductase (34)

and cytochrome oxidase (27, 18, 25, 24). The drug is known to inhibit both aerobic and anaerobic glycolysis (27, 36) and reports of the inhibition of cholinesterases (35), glutamine synthesis (28), D-amino acid oxidases (37), and succinoxidases (38) have appeared in the literature.

Lindan et al (39) have shown that CPZ brings about an inhibition of the rate of incorporation of glycine-1-$C^{14}$ into rat brain cortex proteins, at concentrations of the drug which have no effect on the respiration of the brain. It is known that the incorporation of glycine into tissue proteins is an energy requiring process, and it is assumed that adenosine triphosphate (ATP) is involved, since uncoupling agents inhibit such incorporation. Therefore, the inhibitive effect of CPZ suggests that it brings about its effects by an uncoupling of phosphorylation from oxidation in brain slices.

Various workers have reported inhibition of adenosinetriphosphatases (ATPases) by the drug (18, 40, 35, 38). However, Kirpekar and Lewis found that ATPase activity was stimulated by CPZ at high concentrations (41). Furthermore, Low obtained stimulation of dinitrophenol-induced ATPase with low concentrations of the drug, and inhibition with high concentrations (31). Magnesium-induced ATPase activity was inhibited by the same concentrations of the drug necessary for the stimulation of dinitrophenol-induced ATPase. It is difficult to evaluate the results of in vitro studies, such as those listed above, in relation to the actions of the drug in vivo. Weiner and Huls (42), in 1961, attempted to more closely approximate actual conditions by performing the assays on brain homogenates prepared from normal animals and from

animals which had received very large doses (up to 800 mg./Kg.) of CPZ. They found no changes in the ATPase activity of homogenates from CPZ-treated animals as compared with those from normals.

3. Effects on Nucleotide Metabolism

Magee, Berry and Rossiter (43) studied the effects of CPZ on the incorporation of $P^{32}$ by slices of respiring guinea-pig brain. At low concentrations of the drug ($10^{-4}$M), no significant changes were observed in labelling or concentration of ATP. However, with higher concentrations of CPZ ($10^{-3}$M) there was a considerable fall in both specific activity and concentration of ATP. Grenell et al (44) found increased levels of ATP in brain and lung of rats injected with 10 and 50 mg./Kg. of CPZ, but observed no changes in heart, liver, or kidney. In subsequent work, (45), using slightly different techniques, they obtained higher levels of ATP in rat brain, but were no longer able to detect a difference between control and CPZ-treated rats. Using improved techniques, Weiner and Huls (42) found no differences between the concentrations of ATP in normal and CPZ-treated rats, when the animals were killed by plunging them into liquid oxygen. However, when the unanesthetized rats were killed by decapitation, ATP levels were higher in CPZ-treated animals than in normals. It is suggested that the destruction of labile phosphate compounds, due to the stimulation of the central nervous system by decapitation, is not as rapid in rats pretreated with CPZ.

It is interesting to note that Kirpekar and Lewis (46) observed marked decreases in ATP concentrations in liver and brain of

rats injected with reserpine. They reported little or no change in levels of ATP in skeletal muscle or heart under the same experimental conditions.

4. Effects on Phospholipid Metabolism

Chlorpromazine has been reported to have several different actions on brain lipid metabolism in vitro and in vivo. Magee et al (43) demonstrated an increased incorporation of $P^{32}$ into phospholipids of guinea-pig brain slices respiring in the presence of CPZ at a concentration of $10^{-4}$M. When the concentration of the drug was increased to $10^{-3}$M there was a fall in $O_2$ consumption accompanied by a decrease in the labelling of phospholipids. Similar effects were later observed (47) with guinea-pig brain slices when the radioactive precursors used were glycerol-1-$c^{14}$ or serine-3-$C^{14}$.

In contrast, Christensen and Wase (48, 49) reported that CPZ at a concentration of $10^{-4}$M inhibited the incorporation of fatty acids into phospholipids. The incorporation of $P^{32}$ into phospholipids in brain and liver homogenates was inhibited by concentrations of the drug as low as $10^{-5}$M.

Ansell and Dohmen (50) demonstrated marked depression of phospholipid turnover, in brain tissue of rats, three hours after the administration of (20 mg./Kg.) CPZ. This effect is still apparent at dose levels down to 5 mg./Kg. (51). Similarly, Wase et al. (16) observed reduced activity in the hypothalamus and cortex of rats, 48 hours after they were injected with 50 mg./Kg. of CPZ. However, in the hypothalamus only, there was an increase in the specific activity of lipid phosphorus 24 hours after the injection of the drug.

Furthermore, rats receiving 25 mg./Kg. of CPZ showed little change in lipid phosphorus incorporation after 24 hours and slight increases after 48 hours.

More recent in vivo observations indicate that, with lower doses of CPZ, there is a stimulation of phospholipid synthesis in brain tissue (49). Similarly, Grossi et al. (52) reported increased incorporation of acetate and mevalonic acid into brain phospholipid fatty acids with concentrations of CPZ of $10^{-4}$ and $10^{-5}$M. Liver lipid biosynthesis was not affected under these same conditions. With higher concentrations of CPZ ($10^{-3}$M), inhibition of lipid biosynthesis was observed in both brain and liver. This agrees with the earlier reports by Magee, Berry and Rossiter (43). Grossi et al also reported stimulation of lipid biosynthesis in brain tissue of rats injected with 3.5 mg./Kg. of CPZ, but little or no effect with doses of 35 mg./Kg.

The uptake of $P^{32}$ in rats treated with CPZ was also investigated by Lingjaerde et al in 1958 (53). The total uptake of $P^{32}$ was measured in brain, liver, kidney and adrenals or normal, adrenalectomized and hypophysectimized animals. The administration of CPZ in a dosage of 10 mg./Kg. resulted in increased uptake of $P^{32}$ in all tissues studied. However, since no attempt was made to fractionate the tissues, it is difficult to compare these results with the data reported above.

5. Effects on Adrenal-Pituitary Axis

There are some discrepancies in the reports on the effects of CPZ on the pituitary-adrenal axis. Olling and DeWied (54) reported that the pretreatment of animals with CPZ, inhibited the

release of adreno-corticotrophic hormone which normally occurs during stress. Similar results were reported by Ohler, Sevey and Wiener (55). Clinical studies indicate that pretreatment, with CPZ, of schizophrenics about to undergo insulin coma treatment, resulted in an inhibition of the usual rise in plasma 17-hydroxy-corticosteroids (56). Tui, Riley and Orr (57) found significantly lower values for this steroid fraction in the plasma of patients receiving CPZ. In contrast to these observations, Harwood (58) reported increased levels of 17-hydroxycorticosteroids following administration of CPZ to monkeys, in doses up to 10 mg./Kg. However, in a later study (59) Harwood and Mason showed increased levels of 17-hydroxycorticosteroids with small doses of CPZ (up to 2.5 mg./Kg.) but no stimulation with larger doses.

## C. SURVEY OF TISSUE FRACTIONATION PROCEDURES

There are a number of procedures currently being used for the fractionation and estimation of the various phosphorus (P) containing compounds in biological tissues. Most of these procedures make use of a preliminary fractionation of the tissues into four main groups of compounds; acid soluble P, lipid P, nucleic acid P and phosphoproteins. For most purposes, further separation of the compounds within these groups is desired.

### 1. Acid Soluble P Compounds

Extraction of the tissue with ice cold trichloro-acetic acid (TCA) or perchloric acid ($HClO_4$) removes the acid soluble P compounds. These include inorganic phosphate, creatine phosphate and various acid soluble nucleotides. Procedures for the separation

or estimation of the compounds within this group, particularly the adenosine nucleotides, fall into three general categories; chemical, enzymatic and chromatographic.

Classically, purines and pyrimidines and their derivatives were separated by precipitation with heavy metals or as alkaloidal salts (60). A later development was the separation of the acid soluble P compounds into three fractions by making use of the different solubilities of their barium salts at pH 8.2 (61). A barium insoluble fraction is obtained which contains adenosine triphosphate (ATP) and adenosine diphosphate (ADP) as well as hexose diphosphate and 3-phosphoglyceric acid. Since both ATP and ADP contain adenine, ribose and phosphorus, analyses for these components will indicate the content of ATP and ADP. Inorganic phosphate can be removed by precipitation with calcium or magnesium ions before estimation of ATP or ADP is undertaken. The determination of labile P by hydrolysis with acid, has been used by various investigators (62-65). The terminal phosphate of ATP is split off by hydrolysis, for definite time intervals, with N HCl, $H_2SO_4$ or $HClO_4$. The amount of P liberated by this process is then determined by subtracting the previously determined value for inorganic P from the amount present after hydrolysis. This figure is usually referred to as ATP P, although several other compounds will also be broken down under the conditions of the reaction.

Specific enzymic methods, as proposed by Kalckar (66, 67, 68) and modified by various workers (69-73) have been used to estimate the concentrations of ATP, ADP and AMP in acid extracts of biological tissue. Mackler et al measured the amount of P liberated by the action of the enzyme specific for an individual nucleotide, and used

this as a measure of the concentration of that nucleotide (72). A slightly different approach was that reported by Wiener in 1961 (73). The initial optical density (O.D.) of a solution containing the sample plus appropriate reagents, was recorded at 265 mu, and a solution of adrenylic acid deaminase was added. The resulting O.D. change was an indication of the amount of AMP present in the sample. Similarly, the change in O.D. produced by the addition of myokinase was proportional to one-half the amount of ADP in the sample, and that produced by the addition of hexokinase was proportional to one-half the ADP and all the ATP in the aliquot.

Another enzymic method, specific for ATP, makes use of the linear luminescence response of firefly extract to added ATP, when all other factors are present in excess (74).

Separation of the various acid soluble P-containing compounds has also been accomplished using chromatographic techniques. Hanes and Isherwood, in 1949, were the first to report the separation of these compounds by paper chromatography (75). Since then, numerous reports have appeared, in the literature, describing paper chromatographic methods which are useful in the separation of acid soluble nucleotides (76-84). Many of them make use of the solvents recommended by Hanes and Isherwood, or modifications of them (76, 77, 78, 80, 81, 82, 84). The use of two-solvent, one-dimensional chromatography was suggested by Krebs and Hems (77). Orthophosphate is separated from the adenosine nucleotides by ascending chromatography in the first solvent, and the nucleotides are further separated from one another by descending chromatography, in the opposite direction, with the second solvent. This technique has been used by several workers in subsequent

reports (78, 80, 82, 84). Two-dimensional chromatography, in which the second solvent is run at right angles to the first, has also been advocated (82, 83). A thorough separation of many P-containing compounds has been reported by Gerlach, Fleckenstein, and Gross, using five solvent systems and two-dimensional chromatography (82).[1]

The use of paper electrophoresis has also been described for this purpose (79, 85).

Cohn, in 1950, described a method for the separation of the nucleotides of the nucleic acids, using anion exchange chromatography (86). The principles of this method were applied by Hurlbert et al, (87) to the separation of possible precursors of nucleic acids, in protein-free filtrates of animal tissues. The concentration of the eluent is increased gradually and reproducibly by the use of a mechanical mixing apparatus, the term "gradient elution" being used to describe the process. Modifications of this procedure have been used by a number of workers since then (88-92).

2. Lipid P

Lipid P can be removed by extracting the precipitate remaining after the acid-soluble extraction with organic solvents such as ether, alcohol or chloroform (93). The solutions thus obtained can be used directly to obtain values for the total lipid P, or they can be further fractionated and individual phosphatides estimated. Such additional fractionation has been carried out by subjecting the lipids to mild alkaline hydrolysis and separating the hydrolysis products by paper chromatography (94).

1. See footnote on page 14.

3. Nucleic Acid P

The residue from the lipid extractions may be further extracted to obtain the nucleic acids.

In the Schmidt and Thannhauser method (95) the ribonucleic acid (RNA) is separated from deoxyribonucleic acid (DNA) and most of the tissue protein, by overnight hydrolysis at 37°C with N alkali. Acidification of the alkaline digest with TCA or $HClO_4$ precipitates the DNA and protein which are centrifuged down while the RNA remains in the supernatant as acid soluble nucleotides. The RNA is then determined on the basis of the P content of the supernatant.

With the Schneider procedure (93) the RNA and DNA are extracted together by means of hot TCA or $HClO_4$ thus freeing them from most of the tissue protein which remains insoluble. Since both nucleic acids are hydrolyzed to their constituent nucleotides or free bases, there is no means of separating them. Instead they may be determined on the basis of specific color reactions for pentose and deoxypentose.

Hurlbert and Potter (96) first extracted the nucleic acids from the tissue with 10% sodium chloride at 100°C and then precipitated them overnight with alcohol at 3°C. They were then treated with alkali and P determined as in the Schmidt and Thannhauser method.

Note: Since the completion of this work 2 pertinent references have appeared in the literature concerning the separation of acid soluble nucleotides by paper chromatography:

1. Moscarello, M.A., Lane, B.G. and Hanes, C.S. Canad. J. Biochem. Physiol. 39:1755, 1961
2. Wood, T. J. Chromatography 6:142, 1961

# METHODS

## A. ANIMALS

Animals used were adult, male albino rats of the Sprague-Dawley strain, weighing from 200 to 350 grams. They were kept in large cages for freedom of movement, and were fed ad libitum on Purina fox chow and water.

## B. INJECTIONS

Animals were injected intraperitoneally with either 25 or 50 mg. of CPZ per Kg. of body weight. One hour later they received an intraperitoneal injection of 200 microcuries of radioactive phosphorus ($P^{32}$) regardless of weight. It has been shown that doses much larger than 200 microcuries are necessary to produce metabolic changes in the rat due to radiation (97,98). The administration of the same dose to all animals would tend to produce variations in the activity of the plasma inorganic P, due to differences in dilution of the isotope. For this reason, plasma inorganic specific activities (S.A.) are corrected by multiplying by the body weight of the animal.

Control animals received only the $P^{32}$ injections.

## C. REMOVAL OF TISSUES

The animals were killed 4 hours after the injection of $P^{32}$ in the first series, and 16 hours after $P^{32}$ in subsequent series.

The rats were killed by decapitation, and a blood sample was collected by inverting the body over a small beaker containing a drop of heparin. The blood sample was transferred to a 15 ml. centrifuge tube and kept ice cold until it could be conveniently centrifuged. A vertical incision was made up the abdomen and the

adrenals removed, cleared of adhering adipose tissue and dropped into crucibles standing in dry ice. Approximately 200 to 300 mg. samples of kidney, liver, lung and heart were excised, blotted to remove excess blood, and frozen on dry ice. The brain was removed intact by cutting away the top of the skull, and approximately 300 mg. of tissue, including the hypothalamus-thalamus region, was removed and frozen. All tissues were kept frozen while being weighed and until homogenized.

## D. SEPARATION OF THE PHOSPHORUS COMPOUNDS

### Method 1

Fractionation of the tissues was first accomplished by a chemical separation based upon the procedure of Schneider (93) as modified by Hobbs (62).

Throughout the homogenization and acid soluble fractionation procedures, the tissues and solutions were kept ice cold to eliminate variations due to temperature. Inorganic-P determinations were carried out immediately and while still ice cold. All tissues were treated identically except for quantitative differences in washing procedures.

The general separation scheme is as follows:

1. Plasma Inorganic P

200 microlitres of plasma were removed from the centrifuged blood sample and extracted 3 times with ice-cold 10% TCA. The extracts were combined and an aliquot taken for P estimation.

2. Acid Soluble P

The tissues were homogenized in 10% TCA in a ground glass homogenizer of the Potter-Elvehjem type (99) and transferred to a centrifuge tube. The residue was washed with additional portions of

ice-cold 10% TCA and the extracts were combined. Aliquots of this fraction were then taken for Inorganic P, ATP-P, and Total Acid Soluble P measurements.

3. Inorganic P

Inorganic P determinations were done immediately and while still ice-cold. This is essential to prevent the breakdown of the creatine phosphate, which would occur at higher temperatures, increasing the value for inorganic P. Riedel (100) has shown that the creatine phosphate of liver does not break down under the conditions used and has also shown that there is no detectable creatine phosphate in the adrenal gland.

4. Adenosine Triphosphate (ATP) P

An aliquot from the acid soluble P fraction was hydrolyzed for 20 minutes with 60% perchloric acid ($HClO_4$), which released the two labile phosphates. An inorganic P determination then indicated the total value for inorganic P plus that released from ATP. By subtracting the previously-determined inorganic P, the portion due to ATP was determined. This value was unavoidably increased by the presence of ADP and creatine phosphate which break down under these conditions.

5. Total Acid Soluble P

All of the P in the fraction was released by wet ashing an aliquot from the acid soluble fraction for 10 minutes with 60% $HClO_4$.

6. Lipid P

The residue from the acid soluble extraction was treated with cold alcohol, then boiling alcohol-ether mixture, and finally with ether at room temperature. A portion of the combined extracts was wet ashed with 60% $HClO_4$ and the P estimated.

7. Nucleic Acid P

The residue from the lipid P extractions was further extracted with 10% sodium chloride at 100°C according to the method of Hurlbert and Potter (96). The extracts were combined and the nucleic acids precipitated by the addition of 95% alcohol and storage overnight at 3°C. After washing with alcohol, the precipitate was dissolved and hydrolyzed in 0.1 N sodium hydroxide (NaOH) for 18 hours at 37°C. the addition of cold concentrated HCl to the chilled NaOH extract served to precipitate the deoxyribonucleic acid (DNA) according to the method of Tyner et al (101). The ribonucleic acid (RNA) remained in the supernatant which was removed for P estimation.

After dissolving in NaOH a P estimation was carried out on the DNA fraction.

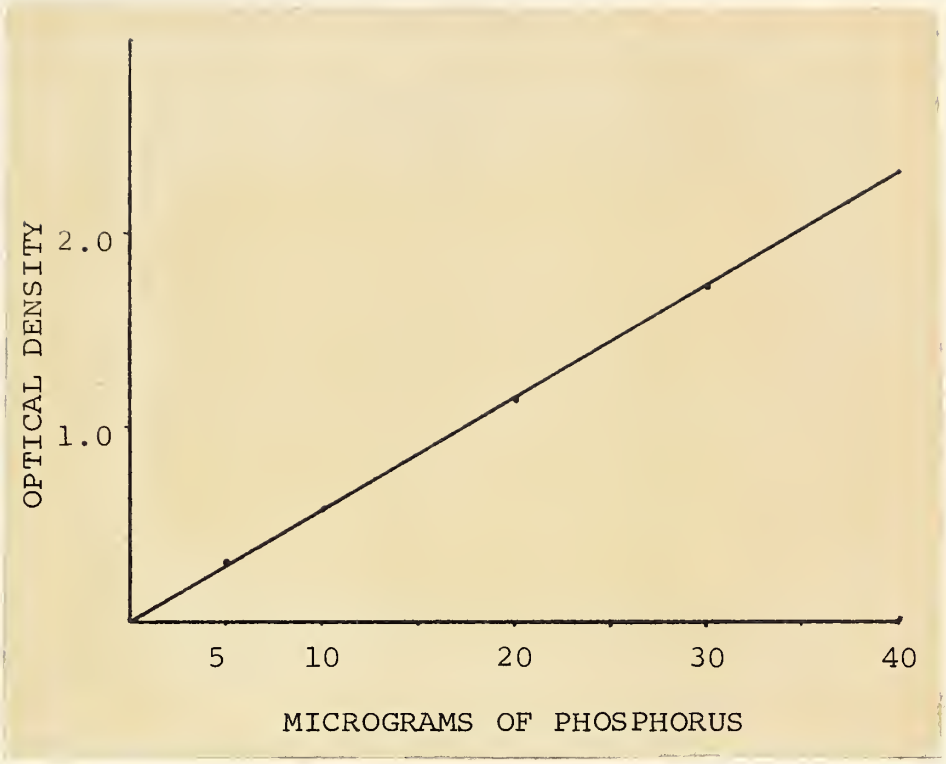
8. Phosphorus Estimation

P was estimated by the method of Ernster, Zetterstrom and Lindberg (102). The P was converted to phosphomolybdate with $HClO_4$ and ammonium molybdate, and then extracted with a mixture of isobutanol and benzene which quantitatively removes the phosphomolybdate. An aliquot was then treated with stannous chloride and the blue color produced was read on a Beckman Model B spectrophotometer at 690 mμ. It can be seen from Figure 1 that Beer's law was satisfied over the range investigated.

9. Radioactivity Determination

The radioactivity of the samples was estimated by placing the blue solution from the P determination into an M-6 liquid Geiger-Müller counter tube (20th Century Electronics) which was connected to a decade scaler (Tracerlab, Inc., Boston, Mass.). Counting was continued until at least 800 counts were obtained which corresponded to an error of not more than 5%.

FIGURE 1




The relationship between optical density at 690 mμ and concentration of P.

(Method of Ernster, Zetterstrom and Lindberg)

Method 2

A preliminary series of experiments was carried out using the above procedures for the separation of the various P fractions. Because of the limitations of this procedure, as far as the accurate separation and measurement of ATP was concerned, and because of the relative importance of the effects of CPZ on this particular fraction, it was decided that a study of more satisfactory separation methods was necessary. Several current methods for the separation and measurement of inorganic P and adenosine nucleotides were examined. The most satisfactory method for our purposes was found to be a modification of the chromatographic procedures of Krebs and Hems (77).

The initial steps in the procedure are similar to those of the previous method.

1. Tissues.

The tissue, after removal from the animal, was immediately frozen on dry ice, weighed, and homogenized in approximately 10 volumes of ice cold 5% TCA. The homogenate was centrifuged while cold, the supernatant removed, and the precipitate saved for the lipid and RNA determinations. The supernatant was then stored at -14°C until used for chromatography. If 0.3 N $HClO_4$ was used instead of 5% TCA, the supernatant was neutralized exactly with concentrated KOH and maintained at near freezing for 30 minutes to precipitate the $KClO_4$. The precipitate was spun down and removed, and the neutralized supernatant retained at -14° for chromatography. Plasma samples were treated similarly.

2. Chromatographic Separation of Acid Soluble P Compounds

Unwashed Whatman No. 1 chromatography papers, 19 cm. by 57 cm., were folded along a line 20 cm. from one end. Suitable aliquots of the

solutions to be analyzed were applied at intervals of about 3 cm. on a line 17 cm. from the same end. By applying a stream of cold air above and below the papers, multiple applications could be made at one spot, until 3 to 10 µg of total P were accumulated at each spot. The plasma acid soluble fraction was found to be free of nucleotides. Therefore authentic ATP, ADP and AMP standards could be added to this fraction before application to the chromatogram.

Glass tanks 30 cm. in diameter were used for both ascending and descending chromatography. They were fitted with a stainless steel framework which supported glass troughs 21 cm. long, holding about 70-80 ml. of solvent.

Two solvents were used. The first recommended by Hanes and Isherwood (75), consisted of 90 mls. isopropyl ether and 60 mls. 90% (w/v) formic acid. The papers were developed first for 4-5 hours by ascending chromatography in this solvent at 20°C. With this solvent the inorganic P was carried up the paper, which the nucleotides remained at or near the starting line. The papers were then removed from the tank and dried in a current of cold air. This usually required about 30 minutes. When dry the papers were spread out flat and the position of the inorganic P was determined by scanning with an end window Geiger-Müller tube, attached to a ratemeter (Tracerlab) which was connected to a recorder (Texas Instruments Inc.). The nucleotides were located by their ultraviolet (UV) absorption with a Mineralight UV lamp (peak emission 254 mu). The portion of the paper containing the inorganic P was then cut off, leaving sufficient paper to form a wick for the descending chromatography with the second solvent.

The papers were reversed, the lower edge serrated with scissors, then developed in the opposite direction by descending

chromatography with the second solvent, consisting of 100 ml. isobutyric acid, 60 ml. N ammonia, and 1.6 ml. of 0.1 M ethylenediaminetetraacetic acid (EDTA). This is the solvent recommended by Krebs and Hems (77). Descending chromatography at 25°C for 18-20 hours results in separation of the various acid soluble nucleotides.

3. Location of Nucleotides

The papers were next removed from the tanks and dried in a fume hood for 2 hours at room temperature. The positions of the nucleotides were located by a modification of the UV photography techniques of Markham and Smith (103). Instead of using photographic negatives as recommended by these workers, negative photocopy papers were used. This eliminated the long developing process required for photographic negatives and also eliminated the necessity of working in total darkness.

The chromatograms and the photocopy papers were fastened together, by means of paper clips, and placed, with the chromatogram upwards, on a flat surface. The UV lamp was suspended 35-40 cm. above the papers, and the negative exposed, through the chromatogram, for 10 seconds. The picture was transferred to positive paper which was then used to locate the positions of the nucleotides on the chromatogram.

By comparison with the standards the areas containing ATP, ADP and AMP were identified, cut out and retained for P estimation and radioactivity determinations.

4. Lipid P

The precipitate from the acid extraction was washed 4 times with approximately 10 volumes of cold 5% TCA, to insure complete removal of the radioactivity associated with acid soluble P compounds. Lipids were then extracted from the precipitate with 95% ethanol at

at 45°C for 30 minutes, followed by ethanol-ether (1-1) at 45°C for 30 minutes, according to the method of Watts and Harris (104). The use of 5 ml. portions of ethanol or ethanol-ether, in the case of the adrenals, and 10 ml. portions with the other tissues, was found to be adequate for complete extraction of the total lipids.

The extracts were combined and aliquots taken for estimation of P and radioactivity.

5. RNA P

RNA P was determined by a modification of the Schmidt and Thannhauser procedure (95) as outlined by Watts and Harris (104).

The residue remaining after the lipid extractions was hydrolyzed with 1 ml. (adrenals) or 4 ml. (other tissues) of 0.3 N KOH for 18 hours at 37°C. The pH was adjusted to 1-2 by the addition of 10 N $HClO_4$ while the temperature was maintained at 0°C. and the resulting precipitate was spun down. The supernatant was the RNA fraction.

Aliquots were taken and analyzed for P and radioactivity.

6. Estimation of P

The areas of the filter paper containing the inorganic P, ATP, ADP and AMP were cut out and wet ashed with 0.7 ml. of a mixture of 3 parts $H_2SO_4$ and 2 parts 60% $HClO_4$. The ashed material was then diluted with 2.0 mls. of water, and neutralized exactly with concentrated $NH_4OH$, using a drop of bromothymol blue as indicator.

The amount of P in each spot was then quantitatively determined by a modification of the method proposed by Lucena-Conde and Prat (105). A single color reagent was used, which contained both acid and molybdate and did not require the use of a reducing agent. It was prepared as follows:

Ammonium molybdate hexahydrate, 8.15 gm. was dissolved in

60 ml. of water. To 30 ml. of this solution, 50 ml. of 12 N HCl and 56 ml. of 36 N $H_2SO_4$ were added slowly. After contraction, 12 N HCl was added to bring the volume back to 136 ml. To 25 ml. of the original molybdate solution 12.5 ml. of 12 N HCl, 12.5 ml. of $H_2O$ and finally 10 ml. of distilled mercury were added. This mixture was shaken for 5 minutes and filtered to obtain 40 ml. of a ruby red solution of molybdenum V. This was then added, with stirring, to the previous acid-molybdate solution and diluted to 200 ml. with water to give the emerald green color reagent. This reagent is stable for at least 6 months (105).

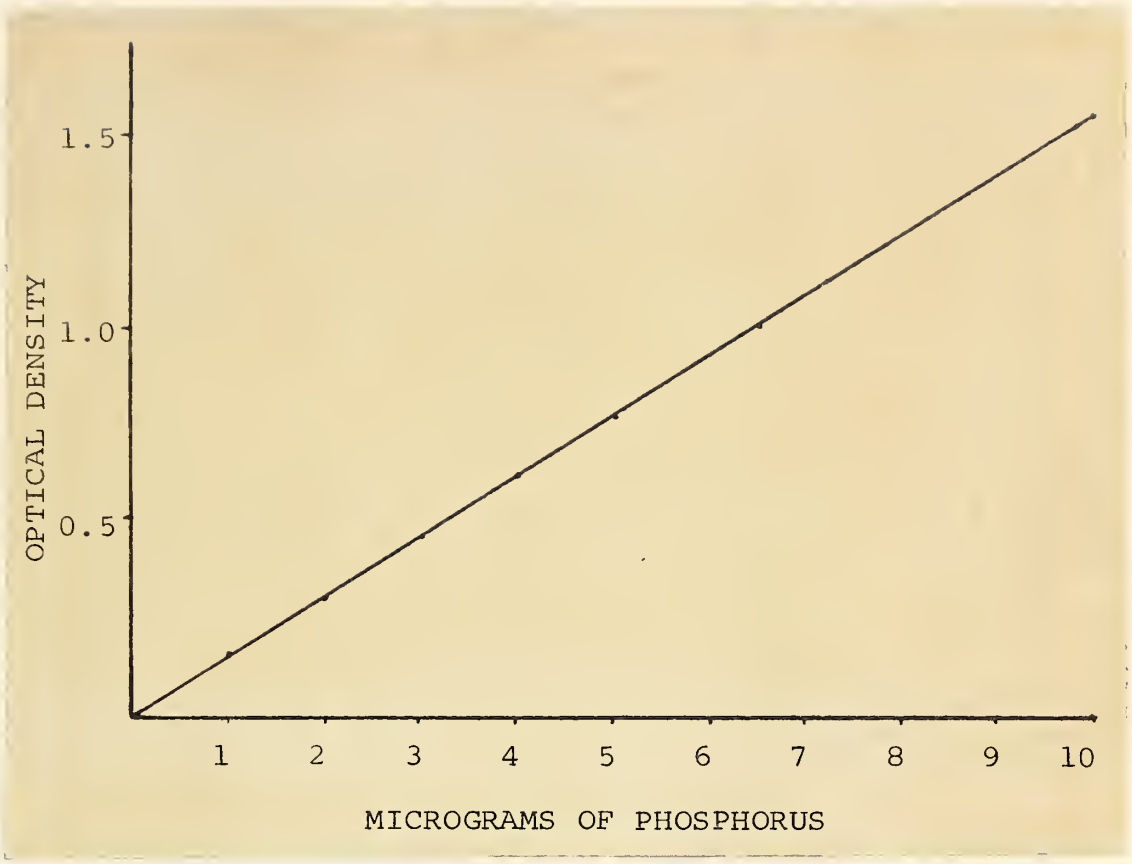
One ml. of the green color reagent was added to the neutralized solution from the wet ashing procedure, and the solution was placed in a boiling water bath for 30 minutes. The solution was then removed, cooled and the absorbance measured on a Beckman DK-2 spectrophotometer at 810 mμ or a Beckman Model B spectrophometer at 760 mμ.[1] Distilled water was used as the reference solution, so that the paper and reagent blank could be determined daily to check that no contamination of paper or utensils had occurred. Blank values were subtracted from each reading before the amount of total P contained in each fraction was calculated.

It can be seen from Figure 2 that Beer's law is satisfied over the range investigated.

The concentrations of P in the lipid and RNA fractions were measured in the same way. Suitable aliquots from these fractions were wet ashed with 1.0 ml. of 60% $HClO_4$ cooled, diluted and neutralized with $NH_4OH$ as before. The green color reagent was added, the solutions were placed in a boiling water bath for 30 minutes, and the P was estimated as described above.

1. The light orange color of the solutions did not interfere with the measurement of the blue color produced by the phosphomolydate.

FIGURE 2




The relationship between optical density at 760 mμ and concentration of P.

(Method of Lucena-Conde and Prat)

7. Radioactivity Determination

The amount of radioactivity in each sample was measured in exactly the same way as described under Method 1. The solutions from the colorimetric determinations were diluted to 10 ml. and their radioactivity determined in an M-6 liquid Geiger-Müller counting tube (20th Century Electronics). They were corrected for background and decay.

In order to correct for variations in the injected dose from experiment to experiment, a sample of $P^{32}$ was measured out, at the time of each series of injections, with the same syringe used for injections, and diluted to 25 ml. in a volumetric flask. Triplicate 20 microlitre aliquots were plated on planchettes and counted with an end-window Geiger-Müller counter tube (Tracerlab, Inc., Boston, Mass.).

A simulated $P^{32}$ standard was also counted each day to check on mechanical variations within the counting equipment.

E. DEFINITION OF TERMS

Specific activity (S.A.) of a sample refers to the number of counts per minute divided by the number of micrograms of total P in that sample.

$$\text{S.A.} = \frac{\text{counts per minute}}{\text{micrograms of P in sample}}$$

Relative specific activity (R.S.A.) refers to the specific activity of a sample expressed as a percentage of the specific activity of the plasma inorganic P for the same animal.

$$\text{R.S.A.} = \frac{\text{S.A. of sample}}{\text{S.A. of plasma inorganic P}}$$

Relative specific activity, relative to the tissue inorganic P (R.S.A.I.), refers to the specific activity of a sample expressed as a percentage of the specific activity of the inorganic P fraction of that same tissue.

$$\text{R.S.A.I.} = \frac{\text{S.A. of sample}}{\text{S.A. of tissue inorganic P}}$$

Corrected specific activity (Corr. S.A.) refers to the plasma inorganic P specific activity of a particular animal, multiplied by the body weight of the animal at the time of killing. This correction is necessary when comparing values for plasma inorganic P specific activity, because of the differences in dilution in the different animals, since all animals received the same dose of $P^{32}$ regardless of their weight. These values are multiplied by a factor of $10^{-3}$ to make them easier to handle.

$$\text{Corr. S.A.} = \text{S.A. of plasma inorganic P} \times \text{body weight} \times 10^{-3}$$

F. STATISTICAL ANALYSIS

Where sufficient figures were obtained to make a statistical analysis valid the calculation was done according to the following formula from Kenney and Keeping (106).

$$t = \frac{[\bar{x}_1 - \bar{x}_2 - (\mu_1 - \mu_2)]}{\hat{\sigma}_{12}}$$

Significant differences at the 95% confidence levels are noted by the use of "S" in the tables.

# RESULTS

## A. EFFECTS OF CPZ ON $P^{32}$ UPTAKE IN THE RAT

Preliminary experiments were carried out as described in the previous section, under Methods. The tissues were fractionated using the procedure described under Method 1.

### 1. 4-hour animals given 50 mg./Kg. of CPZ

Tissues used in this group were brain, lung, liver, kidney, adrenals and heart. They were separated into inorganic P, ATP P, total acid soluble P and lipid P. In some cases the residue remaining after the lipid extractions was wet ashed and assayed for P and radioactivity.

Table 1 presents a comparison between the S.A. of the plasma inorganic P of normal CPZ-treated rats. In the CPZ-treated animals the activity of this fraction was markedly increased over the normals.

When the relative specific activities (R.S.A.) of the other fractions are calculated, the values for the CPZ-treated animals tend to be lower, since they are being divided by larger numbers. Therefore, when the R.S.A. of the two groups are compared, there would appear to be depression of uptake of $P^{32}$ in the treated rats, in all fractions of the tissues studied. This may not be a true indication of the effects of the drug on these fractions, but only a reflection of the higher plasma inorganic S.A. of these animals. Therefore, the R.S.A. has been calculated relative to the tissue inorganic P, which is a more immediate precursor. It is felt that these values (R.S.A.I.) afford a more valid comparison between the normal and treated rats, and they will be referred to in the following discussion of results.

With 4-hour animals, 50 mg./Kg. of CPZ resulted in a marked decrease of $P^{32}$ uptake into tissue inorganic P in all tissues studied, with the

# TABLE 1

## EFFECTS OF CPZ ON SPECIFIC ACTIVITY OF PLASMA INORGANIC P

### NORMALS

| 4-hour animals | | 16-hour animals | |
|---|---|---|---|
| S.A. | Corr. S.A. | S.A. | Corr. S.A. |
| 160 | 43.2 | 53.9 | 14.5 |
| 179 | 35.8 | 73.5 | 21.5 |
| | | 73.9 | 18.8 |
| | | 102 | 20.0 |
| | | 81.6 | 25.5 |
| | | 84.4 | 18.1 |
| | | 150 | 34.5 |
| Mean 170 | 39.5 | 88.5 | 21.8 |

### CPZ-TREATED

| 50 mg./Kg. of CPZ | | | | 25 mg./Kg. CPZ | |
|---|---|---|---|---|---|
| 4-hour animals | | 16-hour animals | | 16-hour animals | |
| S.A. | Corr. S.A. | S.A. | Corr. S.A. | S.A. | Corr. S.A. |
| 258 | 61.9 | 125 | 34.6 | 138 | 43.2 |
| 260 | 56.0 | 114 | 37.6 | 102 | 23.1 |
| 365 | 80.3 | 87 | 25.2 | 196 | 39.6 |
| Mean 294 | 66.4 S | 109 | 32.3 S | 145 | 35.6 S |

S = Significant at the 95% level when compared with the normal animal figures.

exception of the kidney, which was only slightly depressed (Tables 2-7).

Incorporation into ATP P was depressed in lung and heart, stimulated in adrenals and liver, and unchanged in brain and kidney.

The R.S.A.I. of total acid soluble P was essentially unchanged except for the brain tissue where CPZ caused a depression in that fraction also.

When calculated relative to plasma or tissue inorganic P, the incorporation of $P^{32}$ into phospholipids and into the RNA- protein (residue) fraction was markedly decreased.

2. 16-hour animals given 50 mg./Kg. of CPZ

From Table 1 it is apparent that the S.A. of plasma inorganic P was increased in the 16-hour CPZ-treated animals, although the difference was not as marked as it was at 4 hours.

The S.A. of the tissue inorganic P of the treated rats was consistently higher, and the depression of the R.S.A. of tissue inorganic P was not as marked as it was with the 4-hour animals (Tables 8-13). There was little change in this fraction in any tissue with the exception of the adrenals, where a marked decrease was observed.

The incorporation into ATP P of CPZ treated animals was not changed in adrenals, lung, or liver, whereas it was depressed in kidney and heart and stimulated in the brain.

The R.S.A.I. of the total acid soluble P fraction was depressed in the lung, and essentially unchanged in all other tissues studied.

There was a general decrease in the incorporation of $P^{32}$ into phospholipids of CPZ-treated animals. When calculated relative to the plasma inorganic P fraction, all tissues showed marked decreases, and when the calculations were based on the tissue inorganic P fraction, all tissues, with the exception of brain, were decreased.

There was a marked decrease in the incorporation of $P^{32}$ into the RNA P fraction of CPZ-treated rats in all tissues studied. Lung appeared to be most markedly affected and liver was least affected.

3. 16-hour animals given 25 mg./Kg. of CPZ

The tissues studied from this group of animals were adrenals, brain, liver and lung. They were fractionated into inorganic P, lipid P and RNA P only.

The S.A. of the plasma inorganic P fraction of the CPZ-treated rats was increased when compared with the normals. This increase was similar to that observed in rats which had received 50 mg./Kg. of CPZ.

The administration of CPZ in doses of 25 mg./Kg. was found to produce no change in the activity of the inorganic P fraction of the tissues studied (Tables 14-17).

Similarly, the incorporation of $P^{32}$ into phospholipids was not changed by the administration of CPZ at this level. A depression in the activity of the lipid P fraction of brain was observed, when calculated relative to the plasma inorganic P, but it was not significant.

The activities of the RNA P fractions were unchanged in adrenals and brain of rats treated with 25 mg./Kg. of CPZ. Some depression of the R.S.A. of RNA P of lung was observed, but this was not significant. The incorporation of $P^{32}$ into RNA P of the liver was found to be stimulated by this dose of CPZ. The increase in incorporation was significant when calculated relative to the plasma inorganic P, but not when expressed as a percentage of the inorganic P of that same tissue.

# TABLE 2

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF ADRENALS

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 169 | 106 | 158 | 98.9 | 93.5 | 181 | 113 | 107 | 40.6 | 25.4 | 24.0 | | | |
| 247 | 138 | 174 | 97.5 | 70.4 | 171 | 95.5 | 69.2 | 35.8 | 20.0 | 14.5 | 27.1 | 15.1 | 10.9 |
| Mean | 122 | | 98.2 | 82.0 | | 104 | 88.6 | | 23.1 | 19.2 | | 15.1 | 10.9 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 187 | 72.5 | 320 | 124 | 171 | 170 | 66.0 | 91.1 | 17.9 | 6.94 | 9.58 | | | |
| 154 | 59.7 | 216 | 83.1 | 140 | 167 | 64.3 | 108 | 16.6 | 6.39 | 10.8 | | | |
| 208 | 56.9 | 157 | 42.9 | 75.5 | 140 | 38.4 | 67.4 | 25.4 | 6.95 | 12.2 | 9.75 | 2.70 | 4.68 |
| Mean | 63.0 S | | 83.3 | 129 | | 56.2 | 88.9 | | 6.76 S | 10.9 | | 2.70 | 4.68 |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 3

## EFFECTS OF CPZ $P^{32}$ UPTAKE OF LUNG

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 138 | 86.3 | 194 | 121 | 140 | 134 | 85.6 | 97.2 | 33.4 | 20.8 | 24.2 | | | |
| 154 | 86.0 | 101 | 56.5 | 65.6 | 112 | 62.5 | 72.8 | 41.1 | 23.0 | 26.7 | 9.70 | 5.45 | 6.30 |
| Mean | 86.2 | | 88.8 | 103 | | 74.0 | 85.0 | | 21.9 | 25.4 | | 5.45 | 6.30 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Total Acid P | | | | Lipid P | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 185 | 71.8 | 205 | 79.5 | 43.0 | 134 | 52.0 | 72.5 | 24.7 | 9.58 | 13.3 | | | |
| 100 | 38.5 | 272 | 105 | 105 | 99 | 38.2 | 99.0 | 19.2 | 7.40 | 19.2 | | | |
| 157 | 59.1 | 62.5 | 17 | 10.8 | 104 | 28.5 | 66.3 | 30.7 | 8.40 | 19.5 | 5.80 | 1.59 | 3.70 |
| Mean | 56.5 | | 67.2 | 52.9 | | 39.6 | 79.3 | | 8.46 | 17.3 | | 1.59 | 3.70 |
| | | | | | | | | | S | | | | |

S = Significant at the 95% level when compared with the normal animal figures.

TABLE 4

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF KIDNEY

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 188 | 118 | 180 | 113 | 96.8 | 160 | 100 | 85.2 | 49.4 | 30.9 | 26.2 | | | |
| 204 | 114 | 146 | 86.5 | 71.7 | 160 | 89.8 | 78.5 | 47.1 | 26.4 | 23.1 | 23.5 | 13.1 | 11.5 |
| Mean | 116 | | 99.8 | 84.2 | | 94.9 | 81.8 | | 28.6 | 24.6 | | 13.1 | 11.5 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 286 | 111 | 272 | 105 | 95.1 | 236 | 91.5 | 82.5 | 48.6 | 17.2 | 17.0 | | | |
| 261 | 100 | 150 | 57.8 | 57.8 | 178 | 68.5 | 68.5 | 30.1 | 11.6 | 11.6 | | | |
| 366 | 100 | 286 | 78.3 | 78.3 | 255 | 69.7 | 69.7 | 30.6 | 8.4 | 8.4 | 18.2 | 4.90 | 4.90 |
| Mean | 104 | | 80.4 | 77.1 | | 76.6 | 73.6 | | 12.4 S | 12.3 S | | 4.90 | 4.90 |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 5

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF LIVER

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 254 | 159 | 384 | 240 | 151 | 252 | 157 | 99.2 | 68.7 | 42.9 | 27.1 | | | |
| 275 | 154 | 227 | 127 | 82.6 | 223 | 125 | 81.1 | 78.6 | 42.8 | 28.6 | 31.6 | 17.7 | 11.5 |
| Mean | 156 | | 184 | 117 | | 141 | 90.1 | | 42.8 | 27.8 | | 17.7 | 11.5 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 424 | 164 | 374 | 145 | 88.1 | 349 | 135 | 82.4 | | | | | | |
| 194 | 75.0 | 610 | 235 | 318 | 246 | 95 | 127 | 31.4 | 12.0 | 16.3 | | | |
| 387 | 106 | 450 | 126 | 116 | 322 | 88 | 83.1 | 54.9 | 15.0 | 14.2 | 17.4 | 4.8 | 4.5 |
| Mean | 115 | | 169 | 174 | | 106 | 97.5 | | 13.5 S | 15.2 S | | 4.8 | 4.5 |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 6

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF HEART

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 221 | 138 | 214 | 134 | 96.9 | 188 | 117 | 85.0 | 10.7 | 6.70 | 4.84 | | | |
| 260 | 148 | 190 | 112 | 73.2 | 218 | 121 | 83.9 | 11.9 | 6.60 | 4.58 | | | |
| Mean | 141 | | 123 | 85.0 | | 119 | 84.4 | | 6.65 | 4.71 | | | |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 235 | 91.2 | 201 | 78.0 | 85.5 | 194 | 75.1 | 82.6 | 6.22 | 2.40 | 2.64 | | | |
| 209 | 80.5 | 50.1 | 19.3 | 24.0 | 169 | 65.1 | 81.0 | 3.55 | 1.36 | 1.70 | | | |
| Mean | 85.8 S | | 48.7 | 54.8 | | 70.1 | 81.8 | | 1.88 S | 2.17 S | | | |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 7

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF BRAIN

(4-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 16.0 | 10.0 | 12.6 | 7.88 | 7.88 | 15.0 | 9.40 | 9.40 | 0.75 | 0.47 | 0.47 | | | |
| 16.5 | 9.72 | 12.4 | 6.92 | 7.52 | 14.8 | 8.27 | 8.92 | 1.05 | 0.59 | 0.63 | 2.70 | 1.51 | 1.64 |
| Mean | 9.86 | | 7.40 | 7.70 | | 8.83 | 9.18 | | 0.53 | 0.55 | | 1.51 | 1.64 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Total Acid P | | | Lipid P | | | Residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 19.7 | 7.6 | 14.6 | 5.75 | 7.42 | 10.2 | 3.98 | 5.19 | 0.702 | 0.27 | 0.356 | | | |
| 13.4 | 5.2 | 11.4 | 4.40 | 8.51 | 9.1 | 3.50 | 6.78 | 0.246 | 0.09 | 0.184 | | | |
| 19.8 | 5.4 | 13.7 | 3.76 | 6.91 | 13.5 | 3.70 | 6.82 | 0.52 | 0.14 | 0.263 | 2.06 | 0.56 | 1.04 |
| Mean | 6.07 | | 4.64 | 7.61 | | 3.73 | 6.26 | | 0.18 | 0.241 | | 0.56 | 1.04 |
| | S | | | | | S | | | S | S | | | |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 8

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF ADRENALS

(16-hour animals)

### NORMALS

| Inorganic P | | ATP P | | | Acid soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 115 | 213 | 57.1 | 106 | 49.7 | 101 | 187 | 87.8 | 58.4 | 108 | 50.8 | 24.8 | 46.0 | 21.6 |
| 118 | 161 | 67.9 | 92.4 | 57.5 | 103 | 140 | 87.3 | 78.1 | 106 | 66.2 | 22.5 | 30.6 | 19.1 |
| 105 | 143 | 36.8 | 49.8 | 35.0 | 54 | 73.1 | 51.4 | 71.4 | 96.6 | 68.0 | 39.1 | 52.9 | 37.2 |
| 156 | 153 | 70.5 | 69.1 | 45.2 | 131 | 128 | 84.0 | 90.0 | 89,0 | 57.7 | | | |
| Mean | 167 | | 79.3 | 46.8 | | 132 | 77.6 | | 99.9 | 60.7 | | 43.2 | 26.0 |

### CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 135 | 108 | 74.6 | 59.4 | 55.2 | 106 | 84.5 | 78.5 | 38.8 | 30.9 | 28.7 | 12.3 | 9.80 | 9.11 |
| 131 | 115 | 62.6 | 54.9 | 47.8 | 91.7 | 80.1 | 69.7 | 46.3 | 40.4 | 35.3 | 17.8 | 15.6 | 13.6 |
| 91.4 | 105 | 60.3 | 69.3 | 66.0 | 74.8 | 86.0 | 81.8 | 41.4 | 47.6 | 45.3 | 22.2 | 25.6 | 24.3 |
| Mean | 109 | | 61.2 | 56.3 | | 83.5 | 76.7 | | 39.6 | 36.4 | | 17.0 | 15.7 |
| | S | | | | | | | | S | S | | S | |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 9

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF LUNG

(16-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 81.9 | 152 | 65.8 | 122 | 80.3 | 74.8 | 139 | 91.3 | 55.0 | 102 | 67.2 | 30.1 | 55.8 | 36.8 |
| 80.5 | 110 | 60.0 | 81.6 | 74.5 | 88.4 | 120 | 110 | 47.9 | 65.2 | 59.5 | | | |
| 100.3 | 136 | 95.6 | 129 | 95.3 | 62.8 | 85.0 | 62.6 | 55.8 | 75.5 | 55.6 | 25.9 | 35.0 | 25.8 |
| 156 | 115 | 70.5 | 100 | 45.2 | 131 | 97.2 | 84.0 | 90.8 | 58.1 | 58.2 | | | |
| Mean | 128 | | 108 | 73.8 | | 110 | 87.0 | | 75.2 | 60.1 | | 45.4 | 31.3 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 124 | 98.8 | 78.2 | 62.3 | 63.1 | 60.2 | 47.9 | 48.5 | 56.5 | 45.0 | 45.6 | 10.5 | 8.37 | 8.47 |
| 116 | 102 | 81.3 | 71.3 | 70.1 | 80.7 | 70.8 | 69.6 | 60.6 | 53.2 | 52.2 | 5.47 | 4.80 | 4.72 |
| Mean | 100 | | 66.8 | 66.6 | | 59.4 | 59.0 | | 49.1 | 48.9 | | 6.58 | 6.59 |
| | | | | | | | | | S | | | S | S |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 10

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF KIDNEY

NORMALS

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 70.7 | 131 | 55.7 | 103 | 78.8 | 72.6 | 135 | 103 | 55.2 | 102 | 78.1 | 20.5 | 38.0 | 29.0 |
| 81.6 | 111 | 77.4 | 105 | 94.8 | 80.5 | 110 | 98.7 | 53.8 | 73.2 | 65.9 | 12.7 | 17.3 | 15.6 |
| 91.9 | 124 | 74.1 | 100 | 80.6 | 106 | 144 | 115 | 64.2 | 86.8 | 69.9 | 30.5 | 41.0 | 33.2 |
| 118 | 116 | 118 | 116 | 100 | 115 | 113 | 97.5 | 88.2 | 86.5 | 74.7 | | | |
| Mean | 121 | | 106 | 88.6 | | 126 | 104 | | 87.1 | 72.2 | | 32.1 | 25.9 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 143 | 114 | 66.3 | 52.8 | 46.4 | 136 | 108 | 95.1 | 70.6 | 65.3 | 49.4 | 13.2 | 10.5 | 9.23 |
| 131 | 115 | 125 | 110 | 95.4 | 120 | 105 | 91.6 | 65.7 | 57.6 | 50.2 | 20.3 | 17.8 | 15.5 |
| 95.5 | 110 | 43.1 | 49.5 | 45.1 | 87.4 | 101 | 91.5 | 52.1 | 59.9 | 54.4 | | | |
| Mean | 113 | | 70.8 | 62.3 | | 105 | 92.7 | | 57.9 S | 51.4 S | | 14.1 S | 12.4 S |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 11

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF LIVER

(16-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 75.3 | 140 | 71.5 | 133 | 94.9 | 78.7 | 146 | 104 | 96.6 | 179 | 128 | 23.3 | 43.2 | 30.9 |
| 91.8 | 125 | 65.8 | 89.5 | 71.7 | 82.0 | 112 | 89.3 | 85.3 | 116 | 92.9 | 16.0 | 21.8 | 17.4 |
| 86.0 | 116 | 131 | 176 | 152 | 105 | 142 | 122 | 107 | 142 | 124 | 22.1 | 30.0 | 25.7 |
| 113 | 111 | 131 | 128 | 116 | 116 | 114 | 103 | 124 | 122 | 110 | | | |
| Mean | 123 | | 132 | 108.6 | | 128 | 104.6 | | 140 | 114 | | 31.7 | 24.7 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 164 | 131 | 152 | 121 | 92.7 | 151 | 120 | 92.1 | 107 | 85.2 | 65.2 | 27.1 | 21.6 | 16.5 |
| 141 | 124 | 186 | 163 | 132 | 133 | 117 | 94.3 | 116 | 102 | 82.3 | 25.1 | 21.9 | 17.8 |
| Mean | 128 | | 142 | 112 | | 118 | 93.2 | | 93.6 | 73.8 | | 21.8 | 17.2 |
| | | | | | | | | | S | S | | S | S |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 12

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF HEART

### NORMALS

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 100 | 186 | 97.1 | 180 | 97.1 | 90.0 | 167 | 90.0 | 24.8 | 46.0 | 24.8 | 9.86 | 18.3 | 9.86 |
| 107 | 146 | 119 | 162 | 111 | 83.6 | 114 | 78.1 | 23.1 | 31.4 | 21.6 | 7.53 | 10.2 | 7.04 |
| 129 | 175 | 77 | 104 | 60 | 121 | 163 | 93.8 | 20.7 | 28.0 | 16.0 | | | |
| 137 | 134 | 125 | 122 | 91.2 | 138 | 135 | 101 | 29.3 | 28.7 | 21.4 | | | |
| Mean | 160 | | 157 | 89.8 | | 148 | 90.7 | | 33.5 | 20.9 | | 14.2 | 8.45 |

### CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 202 | 161 | 177 | 141 | 87.6 | 187 | 149 | 92.6 | 18.9 | 15.1 | 9.36 | 6.90 | 5.50 | 3.42 |
| 179 | 157 | 117 | 103 | 65.4 | 131 | 115 | 73.2 | 14.1 | 12.4 | 7.88 | 5.91 | 5.18 | 3.30 |
| 146 | 167 | 53 | 62 | 36.3 | 125 | 144 | 85.6 | 20.5 | 23.6 | 14.0 | | | |
| Mean | 162 | | 102 | 63.1 | | 136 | 83.8 | | 17.0 S | 10.4 S | | 5.34 S | 3.36 S |

S = Significant at the 95% level when compared with the normal animal figures.

TABLE 13

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF BRAIN

(16-hour animals)

NORMALS

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 13.5 | 25.0 | 14.6 | 27.1 | 108 | 12.5 | 23.2 | 92.6 | 1.77 | 3.28 | 13.1 | | | |
| 16.4 | 22.3 | 16.1 | 21.9 | 98 | 12.9 | 17.6 | 78.6 | 2.00 | 2.72 | 12.2 | 2.36 | 3.21 | 14.4 |
| 16.4 | 22.2 | 7.98 | 10.8 | 48.7 | 6.45 | 8.73 | 39.3 | 1.54 | 2.08 | 9.39 | | | |
| 19.0 | 18.6 | 23.1 | 22.6 | 122 | 15.2 | 14.9 | 80.0 | 2.37 | 2.32 | 12.5 | | | |
| Mean | 22.0 | | 20.6 | 94.2 | | 16.1 | 72.6 | | 2.60 | 11.8 | | 3.21 | 14.4 |

CPZ-TREATED (50 mg./Kg.)

| Inorganic P | | ATP P | | | Acid Soluble P | | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 16.2 | 12.9 | 36.8 | 29.3 | 227 | 17.7 | 14.1 | 109 | 1.70 | 1.35 | 10.5 | | | |
| 21.3 | 18.7 | 20.1 | 17.6 | 95 | 14.9 | 13.1 | 70 | 1.79 | 1.57 | 8.40 | 0.96 | 0.84 | 4.51 |
| 17.6 | 20.2 | 23.1 | 26.6 | 131 | 17.7 | 20.3 | 101 | 2.36 | 2.71 | 13.4 | | | |
| Mean | 17.3 | | 24.5 | 151 | | 15.8 | 93.3 | | 1.88 | 10.8 | | 0.84 | 4.51 |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 14

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF ADRENALS

(16-hour animals)

NORMALS

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 224 | 149 | 147 | 98.0 | 59.0 | 140 | 93.3 | 65.4 |
| 106 | 130 | 70.4 | 86.3 | 65.6 | 62.9 | 77.1 | 62.5 |
| 143 | 142 | 84.4 | 83.6 | 66.4 | 93.6 | 92.7 | 59.3 |
| Mean | 140 | | 89.3 | 63.7 | | 87.7 | 62.4 |

CPZ-TREATED (25 mg./Kg.)

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 171 | 135 | 114 | 82.6 | 68.4 | 135 | 68.9 | 68.4 |
| 299 | 153 | 125 | 63.8 | 41.8 | 111 | 80.4 | 45.2 |
| 187 | 135 | 117 | 92.1 | 60.9 | 117 | 92.1 | 59.4 |
| Mean | 141 | | 79.5 | 57.0 | | 80.5 | 57.7 |

S = Significant at the 95% level when compared with the normal animal figures.

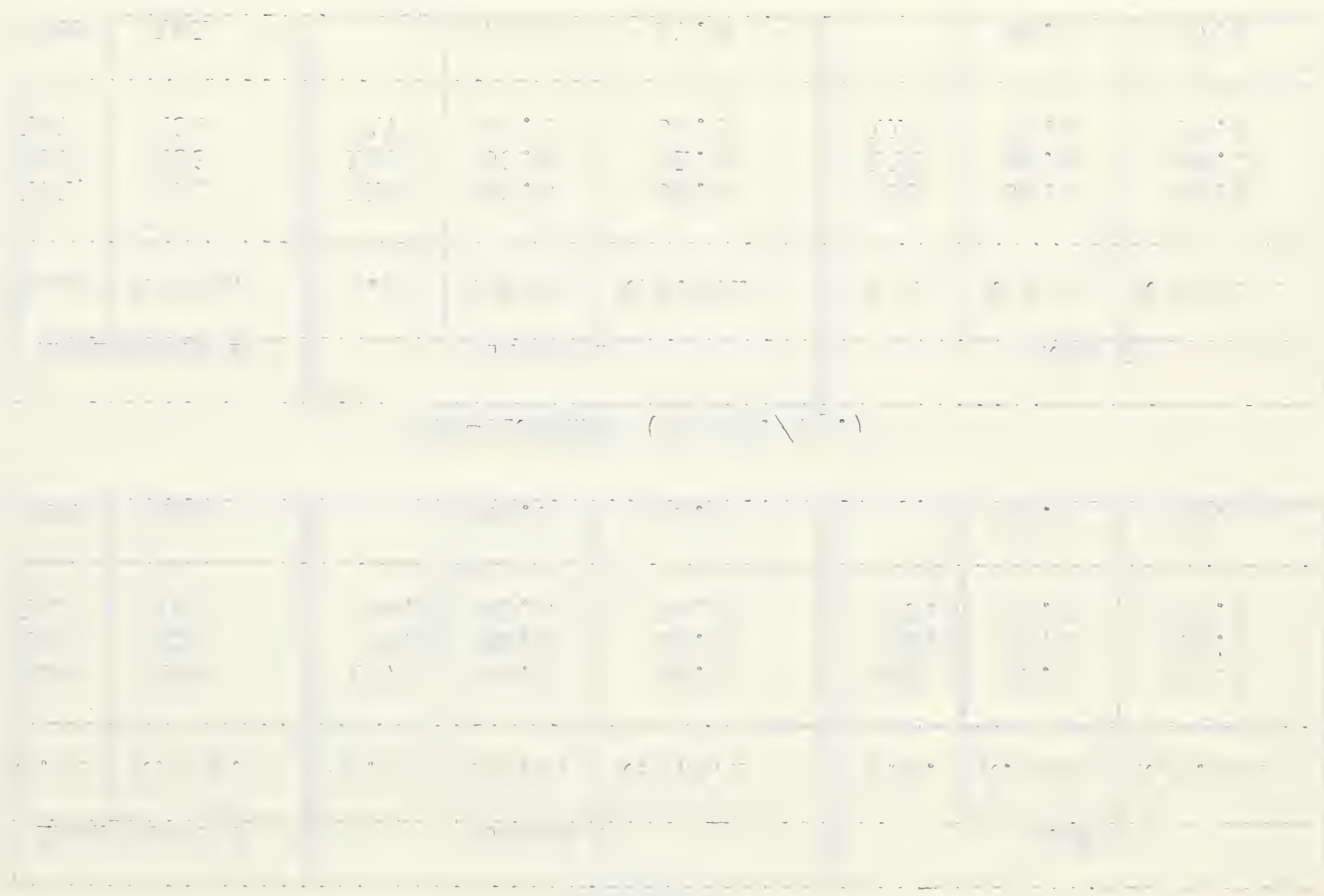


TABLE 15

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF LUNG

(16-hour animals)

NORMALS

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 163 | 109 | 90.6 | 60.4 | 65.5 | 56.6 | 37.7 | 46.5 |
| 106 | 130 | 65.7 | 80.5 | 55.6 | 41.5 | 50.9 | 34.7 |
| 92.5 | 91.6 | 60.6 | 60.0 | 50.5 | 43.0 | 42.6 | 39.2 |
| Mean | 110 | | 67.0 | 57.2 | | 43.7 | 40.1 |

CPZ-TREATED (25 mg./Kg.)

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 155 | 112 | 101 | 73.2 | 56.0 | 64.8 | 33.1 | 32.2 |
| 192 | 98 | 106 | 54.1 | 55.2 | 48.3 | 35.0 | 33.7 |
| 148 | 117 | 82.9 | 65.3 | 65.2 | 47.7 | 37.6 | 31.2 |
| Mean | 109 | | 64.2 | 58.8 | | 35.2 | 32.4 |

S = Significant at the 95% level when compared with the normal animal figures.

# TABLE 16

## EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF LIVER

(16-hour animals)

NORMALS

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 171 | 114 | 181 | 121 | 103 | 47.0 | 31.3 | 24.1 |
| 109 | 134 | 111 | 136 | 106 | 23.6 | 28.9 | 27.5 |
| 109 | 108 | 112 | 112 | 102 | 26.3 | 26.0 | 21.6 |
| Mean | 119 | | 123 | 104 | | 28.7 | 24.4 |

CPZ-TREATED (25 mg./Kg.)

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 173 | 125 | 187 | 136 | 76.5 | 97.3 | 49.6 | 26.7 |
| 254 | 130 | 244 | 124 | 95.7 | 55.2 | 40.0 | 38.2 |
| 166 | 131 | 127 | 100 | 108 | 44.3 | 35.2 | 28.2 |
| Mean | 129 | | 120 | 93.4 | | 41.6 S | 31.0 |

S = Significant at the 95% level when compared with the normal animal figures.

T A B L E 17

EFFECTS OF CPZ ON $P^{32}$ UPTAKE OF BRAIN

(16-hour animals)

NORMALS

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 28.6 | 19.1 | 2.01 | 1.99 | 13.9 | 11.2 | 7.47 | 39.5 |
| 14.5 | 14.4 | 3.31 | 2.21 | 11.6 | 5.69 | 5.63 | 39.2 |
| 21.4 | 26.2 | 2.32 | 2.84 | 10.8 | 4.74 | 5.81 | 22.1 |
| Mean | 19.9 | | 2.35 | 12.1 | | 6.30 | 33.6 |

CPZ-TREATED (25 mg./Kg.)

| Inorganic P | | Lipid P | | | RNA P | | |
|---|---|---|---|---|---|---|---|
| S.A. | R.S.A. | S.A. | R.S.A. | R.S.A.I. | S.A. | R.S.A. | R.S.A.I. |
| 21.8 | 17.2 | 2.50 | 1.97 | 11.5 | 5.87 | 4.62 | 26.9 |
| 26.0 | 18.8 | 2.76 | 2.00 | 9.86 | 11.8 | 6.02 | 33.8 |
| 34.9 | 17.8 | 3.44 | 1.75 | 10.6 | 7.34 | 5.32 | 28.2 |
| Mean | 17.9 | | 1.91 | 10.7 | | 5.32 | 29.6 |

S = Significant at the 95% level when compared with the normal animal figures.

B. SEPARATION OF NUCLEOTIDES

Several methods for the separation and estimation of adenosine nucleotides were investigated. The procedure finally decided upon was a modification of the paper chromatographic techniques of Krebs and Hems (77) as outlined in the previous section under Method 2.

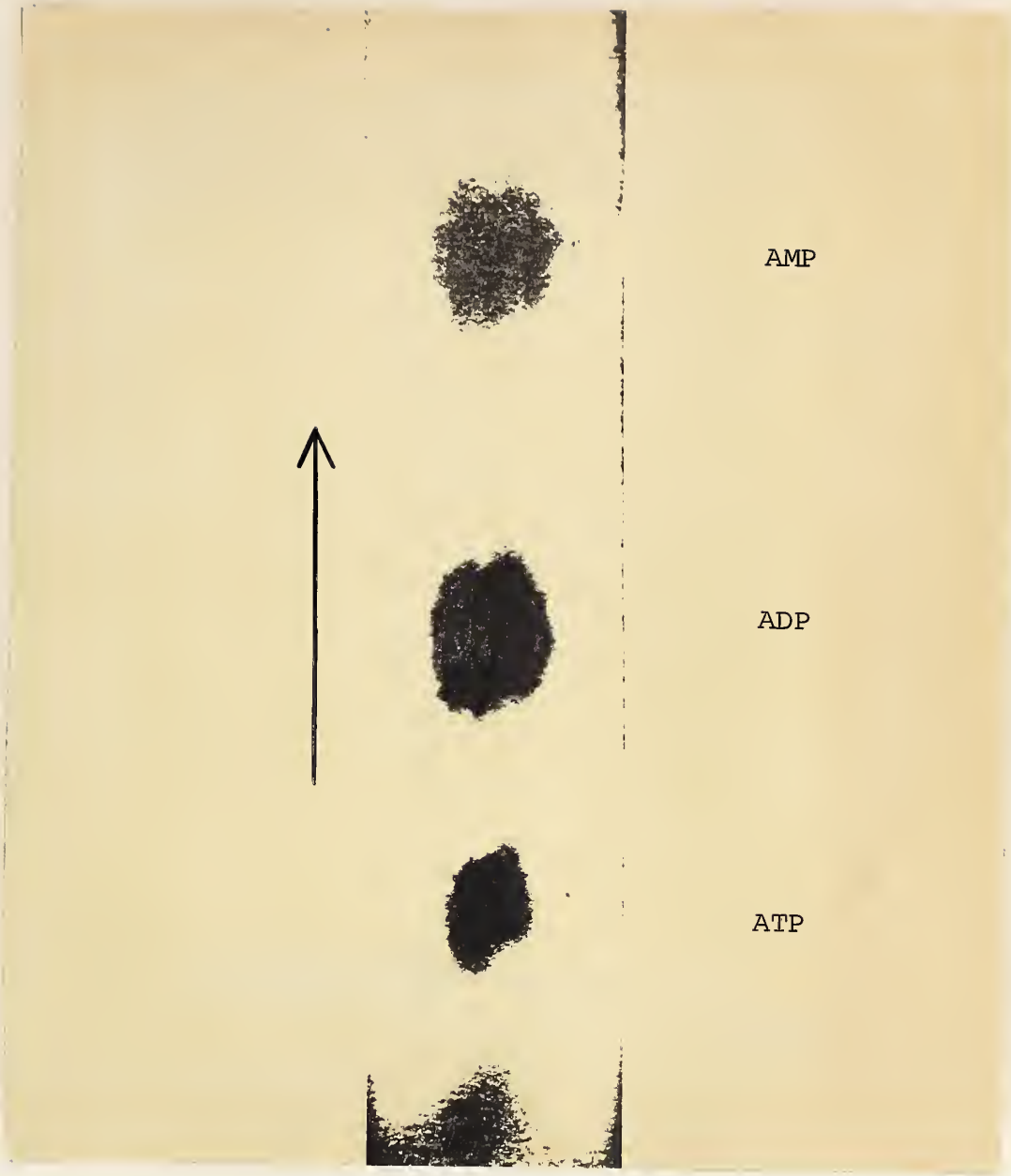
Figure 3 illustrates the separation obtained when a mixture of inorganic phosphate, ATP, ADP and AMP was carried through the procedure. The orthophosphate labelled with $P^{32}$, was first separated from the nucleotides by ascending chromatography in the second solvent. The nucleotides were then further separated from each other by descending chromatography in the second solvent as previously described. Figure 3 is a photostat, taken with UV light, of the chromatogram after development in the second solvent for 18 hours, and shows satisfactory separation of the individual nucleotides. Complete separation of the inorganic P from the nucleotides was obtained, as shown by the fact that the nucleotide-containing areas of the chromatogram were found to contain negligible radioactivity.

The separation obtained when TCA extracts of rat lung and liver were chromatographed, is illustrated in Figure 4. The separation is identical to that obtained with the standards. However, the AMP fraction had a tendency to migrate with the inorganic P in the first solvent. Unless the temperature was carefully maintained between 18-20° C the AMP left the starting point and was lost when the portion of the paper containing the inorganic P was cut away.

The concentrations of the nucleotides were then estimated by wet ashing the filter paper spots and assaying the ashed material

for total P as described in the methods section. Table 18 presents a comparison of duplicate samples of TCA extracts of rat lung and liver tissue, which were carried through the combined procedures. The values obtained for concentration of P and S.A. of the spots are nearly identical.

FIGURE 3




Separation of ATP, ADP and AMP by
Paper Chromatography

FIGURE 4


Separation of Acid Soluble Nucleotides from Rat Tissues by Paper Chromatography

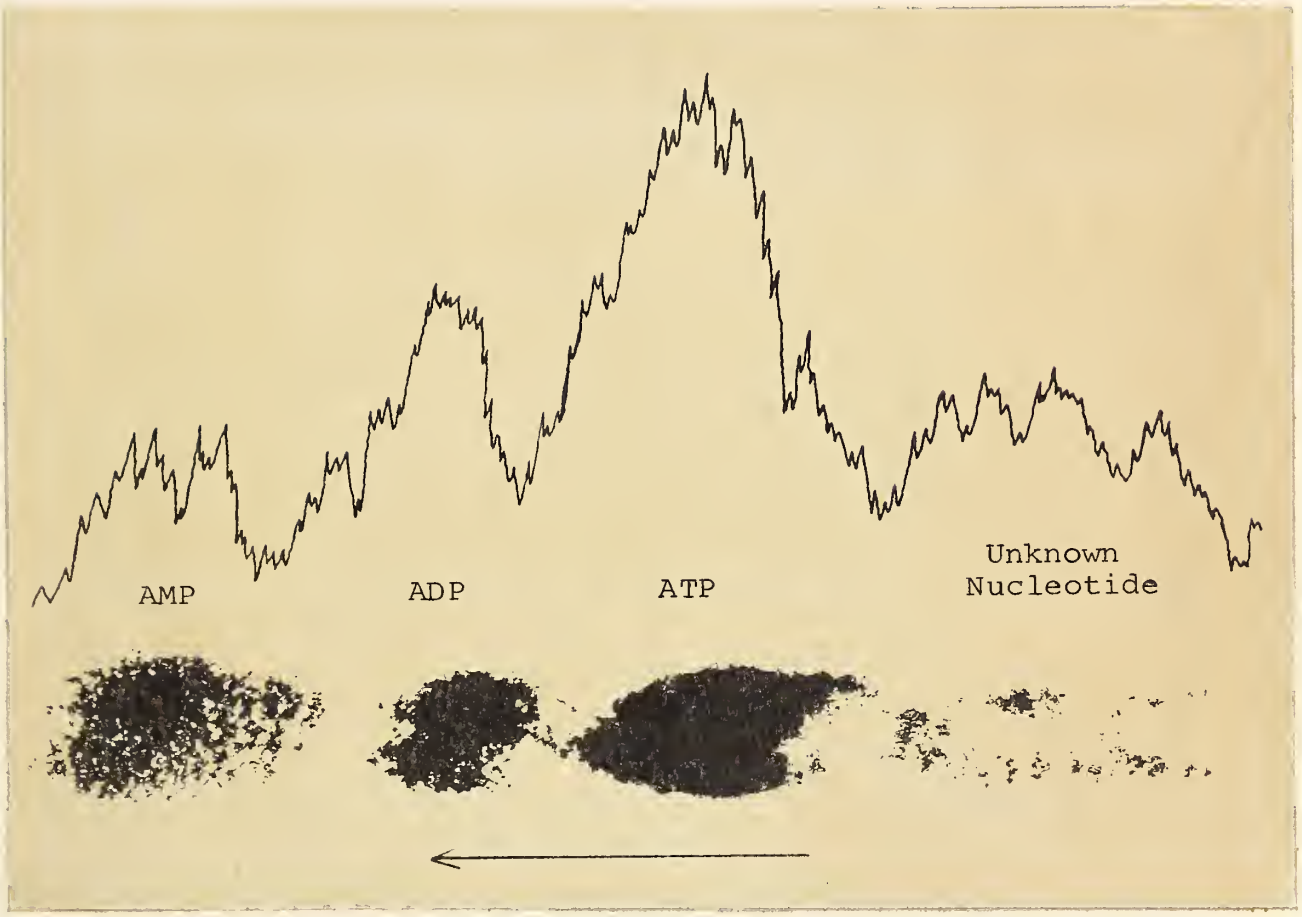
TABLE 18

COMPARISON OF DUPLICATE SAMPLES FROM RAT LUNG AND LIVER AFTER SEPARATION OF NUCLEOTIDES BY PAPER CHROMATOGRAPHY

| | LUNG EXTRACT | | | | LIVER EXTRACT | | | |
|---|---|---|---|---|---|---|---|---|
| | 100 λ Sample 1 | | 150 λ Sample 2 | | 100 λ Sample 1 | | 100 λ Sample 2 | |
| | µg. P | S.A. | µg. P | S.A. | µg. P | S.A. | µg. P | S.A. |
| ATP | 1.40 | 224 | 2.05 | 228 | 0.95 | 296 | 0.98 | 289 |
| ADP | 0.85 | 174 | 1.30 | 169 | 0.85 | 180 | 0.80 | 177 |
| AMP | 0.75 | 121 | 1.05 | 125 | 1.20 | 124 | 1.15 | 129 |

FIGURE 5

DISTRIBUTION OF RADIOACTIVITY ON CHROMATOGRAMS




Tracing made by scanning chromatogram strip with end window Geiger-Müller tube connected to dual recorder

# DISCUSSION

## A. EFFECTS OF CPZ ON P METABOLISM

CPZ in doses of 25 or 50 mg./Kg. appears to produce a considerable increase in the S.A. of plasma inorganic P. This increase is most apparent 4 hours after the injection of $P^{32}$, but is still evident at 16 hours, with both 25 and 50 mg./Kg. of CPZ. Furthermore, the relative specific activities of tissue inorganic P are greatly depressed in the 4-hour animals treated with 50 mg./Kg. of CPZ. This effect is diminished at 16 hours with that same dose, and is absent at 16 hours in rats which had received the smaller dose of CPZ. It would appear that the transfer of $P^{32}$ from the plasma to the intracellular fluid is somehow retarded by the action of CPZ.

Several references have appeared regarding the effects of CPZ on biological membranes (107-111). The drug partially blocks the swelling of frog gastrocnemius muscle, which normally occurs when the muscle is placed in distilled water (107). The metrazol-enhanced passage of dyes into the brain is antagonized by CPZ (108), and the absorption of drugs from subcutaneous depots (109) is also retarded by the drug. Quadbeck and Schmitt (110) stated that neurotoxic substances, including CPZ, decreased the permeability of the blood-brain barrier to phosphates. Spirtes and Guth in 1961, reported inhibition of the swelling of mitochondria from brain or liver, by concentrations of CPZ lower than those needed to affect oxidative phosphorylation or enzyme activity (111). Thus the drug appears to exert an effect on semi-permeable membranes, making them less permeable to water or to substances dissolved in water. This action of CPZ may explain the observed increase in the activity of the plasma inorganic P fraction, since a decrease in the permeability of the cellular membrane would result in such an increased activity.

CPZ in 50 mg./Kg. doses was found to depress the incorporation of $P^{32}$ into phospholipids in all tissues studied. Similar depression of phospholipid turnover with large doses of CPZ has previously been reported by Wase et al (16), Ansell and Dohmen (50), Magee et al (43) and Christensen et al (48). However, the dose of the drug used, is considerably larger than the therapeutic dose, and will also produce many side effects. When the drug was given at a reduced dosage (25 mg./Kg.) the depressant effect on incorporation of $P^{32}$ into lipid P was no longer apparent. This agrees with the reports of Wase et al (16) and Grossi et al (52), but not with the reports of Ansell and Dohmen (50) who obtained a depression with 20 mg./Kg. of CPZ, or Richter (51) who reported inhibition of phospholipid turnover with doses of the drug as low as 5 mg./Kg. Still other reports indicate that low doses of CPZ result in a stimulation of phospholipid turnover (52, 49). It would seem that the effects of the drug on this fraction are variable, depending on the dose used. High doses (50 mg./Kg.) appear to depress the turnover of phospholipids. Lower doses (approximately 25 mg./Kg.) although still well above pharmacological levels, have little or no effect, and doses below 5 mg./Kg. (which are closer to normal therapeutic doses) appear to stimulate phospholipid turnover.

The inhibition of $P^{32}$ incorporation into phospholipids with large doses of CPZ may be related to the uncoupling effect of CPZ on oxidative phosphorylation, which has been demonstrated in vitro (18-27). The biosynthesis of phospholipids is thought to require ATP since it requires conditions in which the rate of oxidative phosphorylation is high (112, 113). Thus, the decreased phospholipid turnover could be due to decreased availability of ATP which could, in turn, be due to the uncoupling effects of CPZ on oxidative phosphorylation. However, it is difficult to relate the actions of CPZ demonstrated in vitro to its

effects in the intact animal. In the first place, the concentrations of the drug used in these in vitro experiments are equivalent to doses larger than normally used therapeutically. The drug is also thought to be taken up from the media and concentrated by the tissues (114, 115, 116). Thus, with normal in vitro techniques, where the drug is added to the media, the actual concentration in the tissue may be higher than the final concentration in the media. Wiener and Huls (42) attempted to get around this difficulty by injecting animals with large doses of CPZ, and using tissues from these animals in their studies. However, they indicate that it is still difficult to be certain of the final concentrations of the drug in the tissue itself, because the addition of the media to the homogenates may tend to dilute the drug from the tissue. A closer approximation to physiological conditions might be obtained if improved methods, such as the Huston-Martin technique (117, 118), were used for the tissue respiration studies. With this method, the tissue is suspended in a gaseous medium, and therefore there would be no dilution of the drug from the tissue.

The incorporation of $P^{32}$ into RNA also seems to be depressed by large doses (50 mg./Kg.) of CPZ. The biosynthesis of RNA is similar to the biosynthesis of phospholipids in that both processes require the presence of high energy phosphate bonds. Therefore if CPZ uncouples oxidative phosphorylation at high doses, it would be expected to inhibit the incorporation of $P^{32}$ into RNA as well as phospholipids. As with the lipid P, this inhibition is noted in all tissues with 50 mg./Kg. of CPZ, but is not apparent with 25 mg./Kg. This may be because CPZ only uncouples oxidative phosphorylation in vivo with doses that are larger than those normally used therapeutically. If this is the case, then it would seem that the pharmacological effects of the drug are not due to its action on oxidative phosphorylation.

The effects of CPZ on other enzyme systems complicates the picture a great deal. Most workers report inhibition of ATPase with low concentrations of the drug (18, 35, 38, 40). This would tend to maintain high concentrations of ATP in the tissues and thus oppose the uncoupling effects of CPZ which would tend to lower the concentrations, Stimulation of ATPase by CPZ has also been reported (41) and Weiner and Huls concluded that the drug has no effect on ATPase at physiological concentrations (42). Weiner and Huls, using advanced techniques, have also reported that in vivo administration of CPZ to rats does not produce any change in the concentration of ATP in brain tissue. This would also indicate that at normal dose levels CPZ does not bring about its effects by an action on phosphorylative metabolism.

Until more definite information is available on the in vivo effects of CPZ, at physiological doses, on both ATPase and oxidative phosphorylation, it will be difficult to evaluate the importance of these effects of CPZ, in relation to the pharmacological actions of the drug.

B. EVALUATION OF METHODS

Of the methods investigated, only the chromatographic techniques seemed to be suitable for adequate separation of the adenosine nucleotides.

The chemical methods are not specific enough for the desired purpose. For example, with the acid hydrolysis method, not only is ATP hydrolyzed but also ADP, hexosephosphate, 3-phosphoglyceric acid and creatine phosphate (61). Errors due to subtraction of the inorganic P from the acid hydrolyzable P also tend to become large.

The separation by precipitation with barium ions is likewise not specific enough. The barium insoluble fraction contains both ATP and ADP. Also, if large volumes of liquid are used, up to 50% of the orthophosphate and ADP present in the original extracts, may be lost in

the barium fractionation (119).

The specific enzymic methods for estimation of adenosine nucleotides (66-73) appear to be rapid and accurate, but they are not suitable for use in radioactive tracer studies, since no actual separation of the individual nucleotides is effected.

Ion exchange chromatography reportedly separates these compounds effectively (86-92), but it requires larger amounts of sample and more elaborate equipment than paper chromatography. Therefore, several current separation methods employing paper chromatography were investigated.

The use of the single solvent recommended by Cerletti et al (79) did not adequately separate inorganic P from the nucleotides. This is an important consideration when working with $P^{32}$ turnover studies, since the S.A. of the inorganic P fraction is relatively high. The first solvent recommended by Hanes and Isherwood (75) was found to satisfactorily separate inorganic P from the organic compounds, but the second solvent recommended by them did not give adequate resolution of the individual nucleotides. A more satisfactory separation was obtained using the second solvent of Krebs and Hems (77). Satisfactory separation of these compounds by paper chromatography has also been reported by Gerlach et al (82) and Tsuboi (83) but the procedures used by these workers require multiple solvents, and are much more time consuming than the methods of Krebs and Hems.

The estimation of the compounds by wet ashing the filter paper spots and estimating the total P present was found to be accurate and reliable. The new spectrophotometric method for the determination of P (105) was quite sensitive and gave reproducible results. It appeared to have some advantages over other methods. The color formed was found to be stable for at least 4 days and samples could be read at different

time intervals without loss of accuracy. This is important when working with large numbers of samples. The method was less time consuming than that of Ernster et al (102) as it was not necessary to extract the solutions with organic solvents such as butanol-benzene in order to reveal the colored complex. The development of the color could be carried to completion in the Kjeldahl flasks originally used for wet ashing, thus reducing the amount of glassware needed. There was only one reagent to prepare and since it was stable for 6 months (105) it could be prepared in quantity and used over a long period of time. It was not necessary to use a reducing agent such as stannous chloride, which normally has to be freshly prepared before each set of determinations.

The estimation of the adenosine nucleotides could also be accomplished by eluting them from the filter paper, and determining their absorbance at 260 mμ. However, since the determination of P was being used in other steps of the procedure (inorganic P and lipid P determinations) it was convenient to adapt this method to the estimation of the nucleotides as well. The calculation of S.A. was also simplified, since values were obtained for both total P and $P^{32}$ in the same sample.

When duplicate aliquots of the same sample were carried through the chromatographic and spectrophotometric determination, the results were nearly identical. The separation of the adenosine nucleotides by paper chromatography and the colorimetric determination of the P content of the separated compounds seems to be a satisfactory method for the desired purposes.

## SUMMARY

1. A preliminary study was made of the effects of CPZ on the $P^{32}$ uptake of a number of P-containing fractions of adrenals, lung liver, kidney, heart and brain of the rat.

2. The P-containing fractions studied were the plasma inorganic P, tissue inorganic P, total acid soluble P, lipid P and RNA P.

3. The administration of CPZ caused significant increases in the $P^{32}$ incorporation of the plasma inorganic P fraction. The changes were greatest 4 hours after the injection of $P^{32}$ but were still evident after 16 hours with either 25 or 50 mg./Kg. of CPZ. It was suggested that the increased activity of the plasma inorganic P was due to a decreased transfer of $P^{32}$ from the plasma to the intracellular fluid. This might, in turn, be due to changes in the permeability of the cellular membrane brought about by CPZ.

4. Decreases in the incorporation of $P^{32}$ into the lipid P and RNA P fractions of all tissues were noted after the administration of 50 mg./Kg. of CPZ. However, when the dose of the drug was reduced to 25 mg./Kg., these decreases were no longer apparent. It was suggested that the uncoupling effects of CPZ on oxidative phosphorylation, which have been demonstrated *in vitro*, are not evident in the intact animal except with doses of the drug larger than normally used therapeutically. Therefore, it is possible that the pharmacological actions of the drug are not due to its effects on phosphorylative metabolism.

5. In view of the relative importance ascribed to the effects

of CPZ on the metabolism of the adenosine nucleotides, an investigation of current tissue fractionation procedures was undertaken.

6. A rapid and convenient method for the separation and estimation of inorganic P, ATP, ADP and AMP was evolved. The separation of the compounds by 2-solvent 2-dimensional paper chromatography was outlined. The use of the method of Lucena-Conde and Prat, for the estimation of total P in the samples, was also described. The advantages of the combined chromatographic and phosphorus estimation procedures, over other current methods, were discussed.

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